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Sporulation Temperature Reveals a Requirement for CotE in the Assembly of both the Coat and Exosporium Layers of *Bacillus cereus* Spores

Christelle Bressuire-Isoard,[a,b] Isabelle Bornard,[c] Adriano O. Henriques,[d] Frédéric Carlin,[a,b] Véronique Broussolle[a,b]

INRA, UMR408 Sécurité et Qualité des Produits d'Origine Végétale, Avignon, France[a]; Avignon Université, UMR408 Sécurité et Qualité des Produits d'Origine Végétale, Avignon, France[b]; INRA, UR407 Pathologie Végétale, Montfavet, France[c]; Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Estação Agronómica Nacional, Oeiras, Portugal[d]

The *Bacillus cereus* spore surface layers consist of a coat surrounded by an exosporium. We investigated the interplay between the sporulation temperature and the CotE morphogenetic protein in the assembly of the surface layers of *B. cereus* ATCC 14579 spores and on the resulting spore properties. The *cotE* deletion affects the coat and exosporium composition of the spores formed both at the suboptimal temperature of 20°C and at the optimal growth temperature of 37°C. Transmission electron microscopy revealed that Δ*cotE* spores had a fragmented and detached exosporium when formed at 37°C. However, when produced at 20°C, Δ*cotE* spores showed defects in both coat and exosporium attachment and were susceptible to lysozyme and mutanolysin. Thus, CotE has a role in the assembly of both the coat and exosporium, which is more important during sporulation at 20°C. CotE was more represented in extracts from spores formed at 20°C than at 37°C, suggesting that increased synthesis of the protein is required to maintain proper assembly of spore surface layers at the former temperature. Δ*cotE* spores formed at either sporulation temperature were impaired in inosine-triggered germination and resistance to UV-C and H_2O_2 and were less hydrophobic than wild-type (WT) spores but had a higher resistance to wet heat. While underscoring the role of CotE in the assembly of *B. cereus* spore surface layers, our study also suggests a contribution of the protein to functional properties of additional spore structures. Moreover, it also suggests a complex relationship between the function of a spore morphogenetic protein and environmental factors such as the temperature during spore formation.

Bacterial endospores are formed in a wide range of ecological niches in soil, as well as in the gastrointestinal tract of invertebrate and vertebrate animals, and in both natural and anthropized environments (1). Physical and chemical conditions prevailing in such niches play a major role in triggering sporulation and in determining the final properties of the resulting spores (2). Laboratory experiments demonstrate the major influence of environment, in particular of temperature, on the efficiency and yield of sporulation, and in spore resistance to wet heat, UV, high hydrostatic pressure, and preservatives or spore response to germinants (3; reviewed in references 1 and 4). Spore resistance and functional properties result from the assembly of several protective structures: cortex, coat, and exosporium. The spore peptidoglycan cortex, a structure common to all endospores, is a major factor in the resistance of spores to heat (1, 4). The cortex is surrounded by a proteinaceous coat, and in organisms such as *Bacillus anthracis* or *Bacillus cereus* the coat is further enveloped by an exosporium, a "balloon-like" structure consisting of a paracrystalline basal layer and an external hair-like nap formed mainly by the collagen-like glycoprotein BclA (5–11). While the coat contributes to protection against peptidoglycan-breaking enzymes, UV light, and oxidative agents, it is also central to the proper interaction of spores with compounds that trigger germination (10). The exosporium also contributes to spore resistance and germination and is additionally a key determinant in the adhesion of spores to cells and abiotic surfaces (11–14).

At the onset of sporulation, the cell divides asymmetrically to produce a larger mother cell and a smaller forespore, or future spore. Each of these cells receives a copy of the genome and deploys cell type-specific programs of gene expression. Assembly of the coat and exosporium takes place in the mother cell and involves the coordinated expression of a large number of mother cell-specific genes, under the governance of the early (σ^E) and late (σ^K) mother cell-specific RNA polymerase sigma factors (10, 11, 15). Studies in the model organism *Bacillus subtilis* have evidenced the role of a group of so-called morphogenetic proteins in directing the assembly of the coat components (10). In *B. subtilis*, the coat is differentiated into an inner layer, an outer layer, and a more external structure called the crust (16–18). Dedicated morphogenetic proteins govern the assembly of the various coat substructures. SafA is the main factor driving inner coat assembly, whereas CotE is the key determinant for the assembly of the spore outer coat and indirectly of the crust through recruitment of the morphogenetic protein CotZ (10). Expression of both SafA and CotE begins early in the other cell line of gene expression, under the control of σ^E, and both proteins localize early to the surface of the

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Address correspondence to Véronique Broussolle, veronique.broussolle@avignon.inra.fr.

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TABLE 1 Strains and plasmids used in this study[a]

Strain or plasmid	Relevant genotype	Source or reference
Strains		
Bacillus cereus ATCC 14579 (type strain)	Wild type; pBclin15 cured	Laboratory collection
B. cereus Δ*cotE*	ATCC 14579 *BC3770*Δ128-313	This work
B. cereus Δ*cotE*Ω*cotE*	ATCC 14579 *BC3770*Δ128-313 + pHT304-18Ω*cotE*	
E. coli DH5α	*fhuA2 lac*Δ*U169 phoA glnV44* Φ80′ *lacZ*ΔM15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Laboratory collection
E. coli SCS110	*rpsL thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ*(lac-proAB)*	
E. coli TOP10	F$^-$ *mcrA* Δ*(mrr-hsdRMS-mcrBC)* Φ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ*(ara leu) 7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
Plasmids		
pdiA	Vector harboring an Sp^r gene	Laboratory collection
pMAD	Ap^r and Em^r shuttle vector	40
pMADΔ*cotE*	Recombinant pMAD harboring *BC3770*Δ128-313	This work
pHT304-18	Ap^r and Em^r cloning vector	73
pHT304-18Ω*cotE*	BC3770 (543 bp) and its upstream region (180 bp) cloned between the SalI and SphI sites of pHT304-18	This work

[a] Sp, spectinomycin; Ap, ampicillin; Em, erythromycin.

developing spore (16, 19–21). SafA is thought to localize close to the spore outer membrane, from where it recruits a group of inner coat proteins. CotE is thought to localize at a distance from the spore outer membrane, at the future inner coat/outer coat interface (16), and, from this position, to recruit the proteins that form the outer coat substructure, as well as CotZ (11, 16, 22). Accordingly, spores of a *cotE* mutant of *B. subtilis* lack the outer coat and the crust (23). Spores of a *cotE* mutant are also impaired in germination and susceptible to lysozyme treatment and to predation by protozoa (23–26).

In spores of *B. anthracis* and the closely related organism *B. cereus*, the coat is thinner and more compact than in *B. subtilis*, but an inner and an outer layer still seem to be present (27). A *B. cereus* mutant unable to produce ExsA, a protein related to *B. subtilis* SafA, forms spores with gross defects in the attachment of the coat and exosporium layers and that are susceptible to lysozyme and impaired in germination (28). Like SafA, ExsA is produced early in sporulation, possibly under the control of σ^E (28). In contrast, CotE, which is also produced early in sporulation, plays a minor role in the formation of the spore coat in *B. anthracis* but a major role in the assembly of the exosporium (29, 30). Although spores of a *cotE* deletion mutant of *B. anthracis* show deficient attachment of the coat to the underlying spore cortex, their main phenotype is a fragmented or entirely missing exosporium (30). Moreover, at least two proteins involved in exosporium assembly, CotY and ExsY (31–34), fail to be assembled in spores of the Δ*cotE* mutant (30). It is inferred that some as-yet-unidentified material fills the space between the coat and the exosporium, or interspace, connecting the two structures; CotE itself may localize at the edge of the coat, helping to connect this material and hence the exosporium to the coat layers (30). While fully heat resistant, spores of a *cotE* deletion mutant were also impaired in germination (30). The Δ*cotE* mutation had no impact on the virulence of *B. anthracis* (30). However, the BclA protein plays a central role in *B. anthracis* pathogenesis by promoting proper interaction of spores with the host phagocytic cell, which allows transport of the spores to sites of spore germination and bacterial growth (35, 36). The role of CotE in the assembly of the spore surface layers in *B. cereus* has not been reported.

Strains of *B. cereus sensu lato* accomplish their life cycles in highly diverse ecological thermal niches, potentially generating spores with highly different properties (37, 38). *B. cereus* is also a major cause of foodborne poisoning, because of the resistance of its spores and its capacity to grow in different kinds of food products. A better understanding of the mechanisms affecting the resistance properties of *B. cereus* spores or their ability to germinate and grow is essential to the design of more-efficient control measures in the food chain.

In this study, we investigated how the sporulation temperature could affect the role of morphogenetic protein CotE in the assembly of the *B. cereus* ATCC 14579 spore surface layers. We constructed a Δ*cotE* deletion mutant, and we studied the consequences of the mutation on the ultrastructure and the protein composition of the spore surface layers and on the functional properties of spores produced at a suboptimal growth temperature (20°C), in comparison to an optimal growth temperature (37°C). We show that deletion of *cotE* affects the composition of the spore surface layers at both 20°C and 37°C, causing the absence or reduced extractability of several proteins and increased representation or extractability of others. The changes in protein composition were greater in spores formed at 20°C. These spores had incomplete and loosely attached coats and exosporium and were susceptible to lysozyme and mutanolysin. Thus, our study unravels an important role of CotE in the assembly of both the coat and exosporium layers in spores of *B. cereus*. Moreover, Δ*cotE* spores show increased resistance to wet heat at 90°C and higher susceptibility to UV-C and to hydrogen peroxide, regardless of the sporulation temperature. Finally, we show that Δ*cotE* spores formed at both temperatures were impaired in inosine-triggered germination and were less hydrophobic than wild-type (WT) spores. While underscoring the importance of CotE in the assembly of the spore surface layers in the pathogen *B. cereus*, our study also documents the complex interplay between the function of a coat/exosporium morphogenetic protein and environmental factors such as temperature during spore formation.

MATERIALS AND METHODS

Strains and general methods. All bacterial strains and plasmids are listed in Table 1. *Bacillus cereus* and *Escherichia coli* strains were grown routinely at 37°C in Luria-Bertani broth (LB) with stirring at 200 rpm. When necessary, antibiotics were added at the following concentrations: ampicillin at 100 $\mu g \cdot ml^{-1}$ for *E. coli* cultures, spectinomycin at 275 $\mu g \cdot ml^{-1}$, and

TABLE 2 Oligonucleotides used in this study

Primer	Sequence[a]
SpcR3′/SpcR5′	GTGGGTAAACCGTGAATATC/TTAATCTGTAGACAAATTGTGA
EavalFNcoI/EavalR	CGATGCATGCCATGGACTACTACCACCATCTCGGC/TTGTCTACAGATTAAGCTCGTGTTATTCAGCCGCC
EamontF/EamontRBamHI	TCACGGTTTACCCACTGGTTAATTACCCAGCACCC/GGCGATATCGGATCCAATGAAAATGTAAAGCGCGC
ConfE1F/ConfE2R	TTCGGATCTTCTACTTGCTC/ATGACATTGTTGAACAAGCG
pMad-ins-down/pMad-ins-up	CATAATTATTCCCCCTAGCTAATTTTCGT/AAGCGAGAAGAATCATAATGGGGAAGG
CotEexFSalI/CotEexRSphI	ATCCTCTAGAGTCGACAAAGGGTGAAACTGGCGC/CCAGTGCCAAGCTTGCATGCCTTATCTCCTATTTCAGGAG

[a] Engineered restriction sites are underlined.

erythromycin at 5 $\mu g \cdot ml^{-1}$ for *B. cereus* cultures. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added to LB agar (LB broth + 15 $g \cdot liter^{-1}$ agar) at a final concentration of 50 $\mu g \cdot ml^{-1}$. *B. cereus* chromosomal DNA was extracted from cells harvested in the mid-log phase of growth in LB (39). Transformation of *B. cereus* was performed by electroporation as previously described (40). Transformants were selected on LB plates containing erythromycin or spectinomycin. All constructions were confirmed by DNA sequencing (GATC, Constance, Germany).

Mutant construction. A *cotE* insertional mutant was constructed by allele exchange as follows. First, fragments corresponding to the regions upstream (578 bp) and downstream (582 bp) of the *cotE* coding region (BC_3370) were PCR amplified from *B. cereus* chromosomal DNA using primer pairs EavalFNcoI/EavalR and EamontF/EamontRBamHI, respectively (the sequences of all primers are given in Table 2), and a 1,197-bp spectinomycin cassette was amplified from plasmid pDiA (41), using primers SpcR3′/SpcR5′. The three PCR fragments were joined together using primers EavalF/EamontR, and the resulting product was introduced into pTOPO-XL (Invitrogen, Saint Aubin, France) to produce pCotE. Next, the fragment was released from pCotE with NotI and BamHI and inserted between the same sites of pMAD (40). The resulting plasmid pMADΔ*cotE* was introduced into *E. coli* SCS110 *dam dcm* mutant (a strain with a double deletion of genes *dam* and *dcm*) to obtain unmethylated DNA to be used for *B. cereus* transformation. pMADΔ*cotE* was introduced into *B. cereus* by electroporation (above), and allelic exchange was selected for by successive cultures at 42°C essentially as described previously (40). White colonies of spectinomycin (Spc)-resistant and erythromycin (Em)-sensitive cells arising from a double crossing-over event were selected on spectinomycin-containing LB agar plates supplemented with X-Gal (50 $\mu g \cdot ml^{-1}$). Deletion of *cotE* was verified by PCR using the following combinations of primers: ConfE1F/ConfE2R, pMad-ins-down/ConfE2R, ConfE1F/pMad-ins-up, ConfE1F/EamontR, EavalF/ConfE2R, pMad-ins-down/pMad-ins-up, pMad-in-down/EamontR, pMad-ins-up/EavalF, and EamontR/EavalF. Deletion of *cotE* was further verified by DNA sequencing of PCR fragments generated with primers ConfE1FSalI and ConfE2RSphI.

For complementation tests, a DNA fragment encompassing the entire *cotE* gene (543 bp) and 180 bp of its upstream region was amplified by PCR with primers CotEexF and CotEexR, cleaved with SalI and SphI, and inserted between the same sites of pHT304-18 (42) to generate pHT304-18Ω*cotE* (Table 1). pHT304-18Ω*cotE* was introduced into the *B. cereus* Δ*cotE* mutant by electroporation followed by selection of transformants on erythromycin-containing LB agar plates.

Spore production. *B. cereus* spores were produced on modified fortified nutrient agar (mFNA) (43). A 0.2-ml volume of an overnight culture in LB at 37°C was spread on mFNA plates and incubated for 7 days at 37°C or for 20 days at 20°C until >95% of cells observed under a phase-contrast microscope at a magnification of ×1,000 (Olympus BX 50; Rungis, France) were free phase-bright spores. Plates were flooded with 4 ml of sterile cold demineralized water, and the spore suspensions were washed in sterile cold demineralized water by a series of four 15-min centrifugations at 4°C (twice at 6,800 × *g*, then at 3,500 × *g*, and finally at 2,200 × *g*). Spores were finally suspended in 2 ml of sterile cold demineralized water. The spore titer in these suspensions was used as an estimation of the sporulation yield on mFNA. For the extraction of spore proteins for mass spectrometry analysis (see below), highly purified spore suspensions were prepared by density gradient centrifugation through a solution of 45% Gastrografin (Bayer, Newbury, United Kingdom). Following centrifugation at 6,800 × *g* for 20 min at 4°C, the spore sediment was washed five times in sterile cold demineralized water (each wash was followed by centrifugation at 4°C for 10 min at 6,800 × *g*) and finally resuspended in 100 μl of phosphate-buffered saline containing 0.1% of Triton X-100 (vol/vol). The presence of 0.1% Triton prevented spore clumping and facilitated the quantification of the suspension and the extraction of the coat proteins (see also below). After heat treatment at 70°C for 15 min, spore suspensions were stored at 4°C or −20°C and used within 1 month.

TEM. Freshly prepared spores were centrifuged at 3,500 × *g* during 5 min and fixed for 1.5 h at room temperature with 2.5% glutaraldehyde (vol/vol) in a 0.1 M sodium cacodylate buffer (pH 7.2) containing 1 $mg \cdot ml^{-1}$ ruthenium red. Spores were washed by three centrifugations (5 min at 3,500 × *g*) in 0.2 M sodium cacodylate and fixed for 1 h at room temperature with 2% osmium tetroxide. Next, the samples were washed with water and mixed with 30% ethanol. After centrifugation, pellets were embedded in 3% (wt/vol) agar and submitted to successive agitated dehydration baths with increasing concentrations of ethanol (50%, 70%, 90%, and 100%). Ethanol was replaced with propylene oxide and sequentially exchanged with araldite resin. Samples were polymerized for 72 h at 48°C. Thin sections (60 nm thick) were obtained with an ultramicrotome (Leica, France) and stained with 2% uranyl acetate and lead citrate. At least 30 spores in each of two independent batches of spores formed at 37°C and 20°C were observed by transmission electron microscopy (TEM; TE Microscope FEI-Philips CM10; Netherlands).

Assessment of spore resistance to physical and chemical treatments. Wet-heat resistance tests at 90°C were performed with 0.2 ml of a heat-activated (10 min at 70°C) spore suspension (10^7 CFU $\cdot ml^{-1}$) in sealed capillary tubes (44). Resistance to H_2O_2 was tested by incubating 1 ml of a heat-activated spore suspension (10^7 CFU $\cdot ml^{-1}$) for 30 min at room temperature in a 5% H_2O_2 solution, as previously described (45). The sensitivity of spores to lytic enzymes was tested by following changes in CFU counts on LB agar plates of spores (10^7 CFU $\cdot ml^{-1}$) exposed for 1 h at 37°C to lysozyme at 7 U $\cdot ml^{-1}$ or mutanolysin (both from Promega) at 250 $\mu g \cdot ml^{-1}$ in water. Lysozyme hydrolyzes the β-1,4 glycosidic bonds between *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlucNAc), while mutanolysin hydrolyzes O-acetylated peptidoglycans. Radiation resistance (45) was assessed by using (i) pulsed-light (PL) equipment (Claranor SA, Avignon, France), delivering 250-μs pulses of broad-spectrum white light (100 to 1,100 nm) under an input voltage of 2,500 V and at fluences ranging from 0.389 to 2.59 J $\cdot cm^{-2}$, and (ii) a device made of low-pressure mercury lamps (model TUV-T8, 15 W; Philips, Netherlands) emitting short-wave UV-C radiations with a peak at 253.7 nm and with an irradiation strength of 1.07 W $\cdot cm^{-2}$. Tests were performed as previously described (45). For each assay, the reduction in viable spore counts was determined and expressed as a percentage ($N/N_0 \times 100$) or as a $\log_{10}$ reduction, $\log_{10}(N/N_0)$, where N_0 is the initial spore count and N is the count of surviving spores. Tests were performed with three independently prepared spore suspensions.

Spore hydrophobicity. A 1.9-ml volume of a spore suspension in water, at an optical density at 600 nm (OD_{600}) between 0.4 and 0.5, was mixed with 100 μl of *n*-hexadecane (Sigma-Aldrich, Zwijndrecht, Neth-

erlands), vortexed for 1 min, and incubated at room temperature for 15 min for separation into solvent and water phases. The percent hydrophobicity was calculated using the following formula: % hydrophobicity = 100 − [(OD_{660} of the spore suspension in water after phase separation/OD_{660} of the spore suspension in water) × 100] (46). Tests were performed with three independently prepared spore suspensions.

Spore germination. Germination assays were performed by incubating heat-activated spores (70°C, 10 min) in a 0.5 mM inosine–10 mM Tris–10 mM NaCl buffer solution or in LB medium at 37°C for 3 h with agitation (200 rpm). Phase-bright (ungerminated) and phase-dark (germinated) spores were scored over time by phase-contrast microscopy. At least 100 spores were counted for each suspension and germination time. The percentage of germination is the number of germinated spores/(total number of spores × 100).

Extraction and analysis of spore proteins. Proteins were extracted from a 200-fold dilution of spore suspensions at an initial OD_{595} of 1.0. Spore suspensions in a volume of 60 μl were mixed with a an equal volume of extraction buffer (4% SDS, 0.125 M Tris-HCl [pH 6.8], 10% 2-mercaptoethanol, 1 mM dithiothreitol [DTT], 10% glycerol, 0.050% bromophenol blue) and heated at 100°C for 8 min. Proteins were electrophoretically resolved on 15% SDS-PAGE gels and visualized by Coomassie brilliant blue R-250 staining. For Western blot analysis, a previously described anti-*B. anthracis* CotE polyclonal antibody was used at a dilution of 1:4,000 (30). A total of 19 bands showing different intensity between the extracts obtained from WT or Δ*cotE* spores formed at 37°C or 20°C were isolated. For liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, gel slices (2 mm by 1 mm) were cut and transferred into a 96-well polypropylene plate, which had been pierced by laser (FrameStar, 0750/Las; 4titude). Gel pieces were washed for 15 min with an acetonitrile–100 mM ammonium bicarbonate mixture (1:1). Digestion was performed for 6 h at 37°C in 50 mM ammonium bicarbonate (pH 8.0), and the quantity of modified trypsin (sequencing grade; Promega) was 0.1 μg per sample (40 μl) (47). The supernatant was transferred by centrifugation in another 96-well plate without holes (FrameStar, 0960/C; 4titude). Peptides were extracted by 5% (vol/vol) formic acid in water-acetonitrile. Tryptic peptides were dried and suspended again in 30 μl of 0.1% (vol/vol) trifluoroacetic acid (TFA) and 2% (vol/vol) acetonitrile. High-performance liquid chromatography (HPLC) was performed on a nanoRsLC system (Dionex, Germany). A 4-μl sample was loaded at 20 μl min^{-1} on a precolumn cartridge (stationary phase, C_{18} PepMap 100, 5 μm; column, 300-μm inner diameter [i.d.], 5 mm; Dionex). Separation was performed on a PepMap 100 C_{18} column (3 μm; column, 75-μm i.d., 150 mm; Dionex). Buffers A and B were as follows: A, 0.1% HCOOH and 2% ACN; B, 0.1% HCOOH and 80% ACN. The peptide separation was achieved with a linear gradient from 0 to 36% buffer B for 42 min at 300 nl min^{-1}, including the regeneration step at 100% buffer B and the equilibration step at 100% buffer A. Eluted peptides were analyzed online with an LTQ-Orbitrap mass spectrometer (Thermo Electron, USA) using a nanoelectrospray interface. Peptide ions were analyzed using the Xcalibur 2.07 software with the following data-dependent acquisition steps: step 1, full MS scan in orbitrap mass-to-charge ratio (*m/z*), 300 to 1,600, profile mode; step 2, MS/MS in linear trap (qz = 0.25; activation time, 30 ms; collision energy, 45%; centroid mode). Step 2 was repeated for the four major ions detected in step 1. Dynamic exclusion time was set to 90 s. A database search was performed with the X!Tandem software (http://www.thegpm.org/tandem/index.html, version Sledge Hammer 2013.09.01) using X!TandemPipeline (version 3.3.3) developed by the PAPPSO platform (http://pappso.inra.fr/index.php). Enzymatic cleavage was declared as a trypsin digestion with one possible miscleavage. Cysteine carboxyamidomethylation and methionine oxidation were set to static and possible modifications, respectively. Precursor mass and fragment mass tolerance were 10 ppm and 0.5 Da, respectively. A refinement search was added with similar parameters except that semitryptic peptide and possible N-ter protein acetylation were searched. The Uniprot database was used with *B. cereus* strain ATCC 14579 (5,363 entries, version 2015/02/19 from http://www.uniprot.org/uniprot). Only peptides with an E value lower than 0.1 were kept, and filters for X!Tandem pipeline were as follows: (i) a minimum of two different peptides was required with an E value smaller than 0.05, and (ii) a maximal protein log (E value) (calculated as the product of unique peptide E values) to conserve a protein was −2. To take redundancy into account, proteins with at least one peptide in common were grouped. This allowed grouping of proteins of similar function. Within each group, proteins with at least one specific peptide relative to other members of the group were reported as subgroups.

Statistical analysis. Results were subjected to a one-way variance analysis to test the effects of the strain-sporulation temperature combination. The significance at P values of <0.05 of the statistical differences of the means was tested with Tukey's test (SPSS Systat version 9, Chicago, IL, USA).

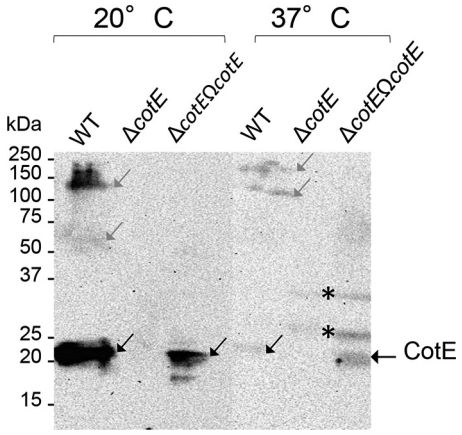

FIG 1 Western blot analysis of protein extracts prepared from spores of *B. cereus* ATCC 14579 (WT) and congenic Δ*cotE* and Δ*cotE*Ω*cotE* strains formed at 20°C and 37°C. Total spore proteins were extracted from the same amount of spores, fractionated by SDS-PAGE, and subjected to Western blot analysis using an anti-CotE polyclonal antibody (30). Black arrows indicate the CotE position, and gray arrows indicate CotE multimers. The asterisks indicate nonspecific bands.

RESULTS

Deletion of *cotE* strongly impacts the structure of the outer layers and the protein composition of *B. cereus* spores formed at 20°C. The role of CotE protein in *B. cereus* spores and of its modulation by sporulation temperature was studied with a Δ*cotE* mutant and a complemented strain (designated Δ*cotE*Ω*cotE* strain) in the background of the ATCC 14579 type strain (wild type [WT]). Both strains sporulate at both temperatures, with a lower titer, however, of Δ*cotE* spores (see Table S1 in the supplemental material).

We started this investigation by examining whether the levels of CotE differed in spores of *B. cereus* ATCC 14579 formed at 20°C and at 37°C. Proteins were extracted from equal numbers of spores in highly purified spore suspensions, resolved by SDS-PAGE, and subjected to immunoblotting with an anti-CotE polyclonal antibody (30). CotE was detected with an apparent mass of 22 kDa, in agreement with the calculated mass of CotE (20.3 kDa) in extracts from WT spores produced at either 20°C or 37°C (Fig. 1). Additional bands around 60 kDa and just below the 150-kDa marker were also observed in the extracts from WT spores, at both temperatures (Fig. 1). Presumably, these species correspond to CotE multimers, as they are absent from extracts prepared from spores of the Δ*cotE* mutant, formed at 20°C or 37°C (Fig. 1). Sur-

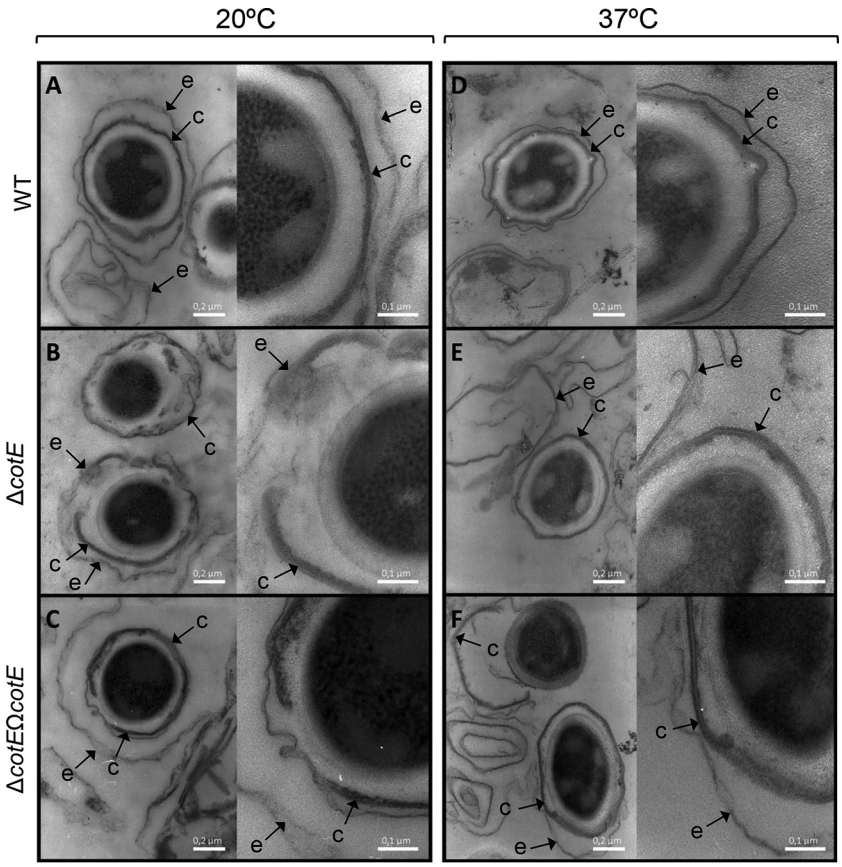

FIG 2 Transmission electron microscopy of thin sections of spores of *B. cereus* ATCC 14579 WT (A and D), Δ*cotE* (B and E), and Δ*cotE*Ω*cotE* (C and F) strains, formed at 20°C (A, B, and C) and 37°C (D, E, and F). c, spore coats; e, exosporium. The concentric layers of WT spores are intact and surround spores at both sporulation temperatures (A and D). Δ*cotE* spores formed at 20°C and 37°C exhibit a fragmented and detached exosporium (B and E), and the coat layers are locally interrupted (B). At both temperatures, some Δ*cotE*Ω*cotE* spores exhibit the same concentric arrangement of the spore layers as WT, but some lack an intact exosporium and surrounding coats (C and F).

prisingly, the level of CotE, either the monomer or the presumed multimers, was 10 times higher in the extracts from spores formed at 20°C than in the extracts obtained from spores formed at 37°C (Fig. 1). The level of CotE was lower in the complemented strain than in the WT at the same sporulation temperature; however, as judged by protein levels, complementation seemed comparatively more efficient at 37°C (Fig. 1). These observations suggest that the sporulation temperature has an impact on the level of spore-associated CotE, or otherwise on its extractability.

To investigate whether the morphology of spores was also affected by the sporulation temperature, purified spores formed at 20°C and 37°C were observed by thin-sectioning TEM. The results, shown in Fig. 2, revealed that the outer layers of WT spores were similar when spores were formed at 20°C (Fig. 2A) or at 37°C (Fig. 2D), with an exosporium forming a continuous structure around the entire spore and with a well-defined coat. In contrast, misassembled coats in addition to an incomplete and/or detached exosporium were observed in Δ*cotE* spores formed at 20°C (Fig. 2B). Spores of the Δ*cotE* mutant formed at 37°C showed an exosporium that either was not attached to the underlying coat or was interrupted at several locations (Fig. 2E). However, the thin coat apposed to the cortex is clearly visible in Δ*cotE* spores formed at 37°C (Fig. 2E) and did not appear to differ from that of the WT (Fig. 2D). The complemented strain produced two kinds of spores at both sporulation temperatures (Fig. 2C and F): some showed a coat and exosporium similar to those of the WT, while other spores had an incomplete and detached exosporium similar to that of Δ*cotE* spores formed at 37°C. More Δ*cotE*Ω*cotE* spores with exosporium and coat defaults were observed at 20°C (Fig. 2C) than at 37°C (Fig. 2F), suggesting that complementation was less efficient during sporulation at 20°C. Together, these results show that the function of CotE becomes more important when spores are formed at 20°C, conditions under which CotE has a role in formation of both the coat and exosporium structures. At 37°C, however, the main function of CotE is in the assembly of the exosporium. Possibly, the increased levels of CotE found in spores formed at 20°C relative to those formed at 37°C reflect the additional function of the protein in the assembly of the coat layers.

Deletion of *cotE* alters the protein composition of *B. cereus* spores formed at 20°C and at 37°C. To determine whether these morphological alterations were accompanied by changes in the composition of the coat and exosporium layers, extraction of proteins from total purified spores was performed before electrophoretic resolution. Coomassie brilliant blue staining of the total spore protein extracts revealed that the intensity of 7 species in spores formed at 20°C and of 3 species in spores formed at 37°C varied between the extracts obtained from Δ*cotE* and those from WT spores (Fig. 3, arrows). These intensity changes may result

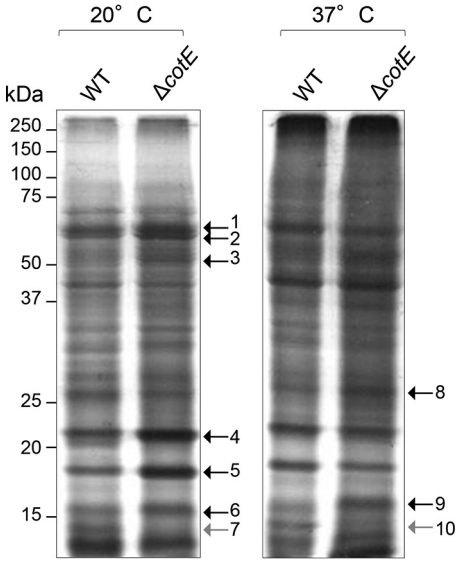

FIG 3 SDS-PAGE of the proteins extracted from the surface layers of *B. cereus* ATCC 14579 WT and Δ*cotE* spores. Coat and exosporium proteins were extracted from spores formed at 20°C and 37°C, separated on a 15% polyacrylamide gel, and stained with Coomassie brilliant blue. Arrows and numbers indicate bands in the protein extract of Δ*cotE* spores with enhanced (black arrows) or lowered (gray arrows) intensity compared to WT.

from altered levels and/or extractability of specific proteins. The proteins present in these bands were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (Table 3). Nineteen proteins were not detected in Δ*cotE* versus WT spores formed at 20°C. Among these proteins were CotJC, previously assigned to the coat and exosporium (48), the exosporium protein CotB (48), a superoxide dismutase (SOD) (BC_1468), and BC_4419, an orthologue of the *B. subtilis* YhcN spore protein (49). In addition, five proteins were detected only in Δ*cotE* spores formed at 20°C, including another superoxide dismutase, BC_5445 (Table 3). Thus, when spores are formed at 20°C, deletion of *cotE* affects the assembly and/or extractability of proteins thought to be present in both the coat and exosporium layers, consistent with the impaired formation of both the coat and exosporium layers as seen by TEM analysis.

Three proteins were not detected in Δ*cotE* spores formed at 37°C (Table 3), including an uncharacterized protein, BC_0212, homologue to *B. subtilis* YusW, BC_5391, an orthologue of the spore coat protein GerQ/YwdL of *B. subtilis* (63% sequence identity) previously shown to be required for proper assembly of the exosporium in *B. cereus* (12) and a [Cu-Zn] superoxide dismutase, homologue to the *B. anthracis* SodC (50). All three were detected in WT spores. Thus, when spores are formed at 37°C, deletion of *cotE* impairs the assembly of at least one protein required for exosporium assembly. This is consistent with the electron microscopy analysis (see above) and suggests that during sporulation at 37°C the main function of CotE is in the proper assembly of the exosporium.

Spore hydrophobicity and germination are affected by *cotE* deletion and not by sporulation temperature. The results described above suggested that sporulation temperature and deletion of *cotE* affected the composition and structure of the spore surface layers. We therefore tested whether the sporulation temperature, the *cotE* deletion, and the combination of the two factors had also an impact on spore properties that rely on proper assembly of the surface layers. One of these properties is spore hydrophobicity. It is well established that impaired formation of the exosporium strongly reduced the hydrophobicity of spores (28). An assay based on the partitioning of spores purified through extensive water washing between aqueous and hexadecane phases was used to screen a library of transposon mutants for those forming spores with reduced hydrophobicity, leading to the identification of the *exsA* and *exsY* genes (28, 31). A similar assay was employed here. Spores produced by the Δ*cotE* strain at 20°C were significantly ($P < 0.05$) less hydrophobic than WT spores (43% versus 79% of spores partitioned into the solvent after phase separation) (Fig. 4). Moreover, an adhesion test showed that Δ*cotE* spores were less adherent to polypropylene tubes than the WT spores, which is in accordance with their lower hydrophobicity (see Fig. S1 in the supplemental material). The same difference in the hydrophobicity of the mutant and the WT was observed when spores were formed at 37°C (87% for the WT versus 44% for Δ*cotE* spores). This suggests that the absence of CotE has a major effect on spore hydrophobicity, while the sporulation temperature does not. Complementation partially restored the WT phenotype, but only when Δ*cotE*Ω*cotE* spores were formed at 37°C.

The spore surface layers also influence the response of spores to germinants (51). *B. subtilis* mutants with strong defects in coat assembly are impaired in germination (11, 52), and *B. anthracis* or *B. cereus* mutants with defects in exosporium assembly are also impaired in spore germination (12, 28, 30, 53). In particular, a *cotE* mutant of *B. anthracis* was shown to be impaired in germination (30). After 1 h in the presence of inosine, 90% of WT spores formed at 20°C had germinated compared to only 26% of Δ*cotE* spores (Fig. 5). The extent of spore germination rate did not increase further with time. Thus, under conditions where deletion of *cotE* impairs assembly of both the coat and the exosporium, the mutation also strongly affects spore germination. However, the extent of inosine-triggered germination after 1 h for Δ*cotE* and WT spores formed at 37°C was similar to that observed for spores produced at 20°C (28% for the mutant and 94% for the WT) (Fig. 5). This suggests that impaired germination may result from altered assembly of specific coat/exosporium components, rather than being caused by the morphological alterations detected by TEM, particularly at 20°C. Complementation partially restored the WT phenotype when spores were formed at 37°C (52% germination for Δ*cotE*Ω*cotE* spores after 1 h and 84% after 3 h at 37°C) (Fig. 5). Complementation was, however, more efficient for Δ*cotE*Ω*cotE* spores formed at 20°C; these spores germinated more rapidly than spores formed at 37°C (83% germination for Δ*cotE*Ω*cotE* spores after 1 h and 95% after 3 h) (Fig. 5). Thus, the germination defect of Δ*cotE* spores was due mainly to deletion of *cotE*, which strongly impacted the germination capacity at the two sporulation temperatures. The percentage of inosine-triggered germination of Δ*cotE* spores remained unchanged between 3 and 24 h after induction whatever the temperature (data not shown). Interestingly, the germination defect detected for Δ*cotE* spores in inosine buffer was not observed when germination was induced in LB broth: more than 90% of spores germinated 1 h after induction, and WT, Δ*cotE*, and Δ*cotE*Ω*cotE* spores behaved very similarly (data not shown). This is in line with the inference that the germination defect imposed by the mutation may be due to im-

TABLE 3 *B. cereus* ATCC 14579 proteins with differential detection in spores of the WT and Δ*cotE* strains formed at 20°C and 37°C[a]

Protein identification[b] in spores formed at temp of:	Locus (gene name)	Protein accession no.	Mol mass (kDa)	Protein E value[c]	% of sequence covered	No. of spectra[d]	Detection in WT spores	Detection in Δ*cotE* spores	Putative location of protein in spore[e]
20°C									
CotJC protein	BC_0821	Q81HI7	21.6	−63.25	38	24	+	−	Coats and exosporium
Spore coat protein B	BC_0390	Q81IJ6	16.7	−24.03	24	11	+	−	Coats and exosporium
Superoxide dismutase	BC_1468	Q81FV0	26.7	−28.04	23	10	+	−	Exosporium
Uncharacterized protein	BC_4419 (*yhcN*)	Q817V9	24.4	−43.80	47	15	+	−	Coats
Nucleotide-binding protein	BC_1159	Q81GN2	18.3	−30.49	42	12	+	−	ND
Transcription elongation factor	BC_4374	Q817Z6	17.4	−39.00	56	11	+	−	ND
Peptidyl-prolyl *cis-trans*-isomerase	BC_4061	Q819C5	15.7	−21.45	43	10	+	−	ND
Uncharacterized protein	BC_4047 (*cotα*)	Q819D8	14.0	−59.85	68	21	+	−	Coats and exosporium
Phosphoenolpyruvate	BC_4048	Q819D7	62.6	−82.05	30	25	+	−	Cytosolic fraction
DEAD box ATP-dependent RNA helicase CshA	BC_0259	Q81IT9	59.3	−45.90	24	17	+	−	ND
Pyruvate synthase alpha chain	BC_3774	Q81A21	64.3	−40.39	20	12	+	−	Cytosolic fraction
2,3-Bisphosphoglycerate-independent phosphoglycerate mutase	BC_5136	Q815K7	56.2	−25.80	18	11	+	−	ND
Pyruvate oxidase	BC_2328	Q81DM9	61.8	−30.12	17	11	+	−	ND
Long-chain-fatty-acid-coenzyme A ligase	BC_4526	Q817L4	55.2	−33.46	19	10	+	−	Cytosolic fraction
Adenylate kinase	BC_0152	Q81J22	23.7	−26.99	50	13	+	−	ND
Nitroreductase family protein	BC_3390	Q81B06	22.6	−48.24	46	26	+	−	Cytosolic fraction
Dipicolinate synthase subunit B	BC_3800	Q819Z6	21.6	−48.93	56	17	+	−	Cytosolic fraction
FMN-dependent NADH-azoreductase 1	BC_1835	Q81EX6	22.9	−27.02	36	12	+	−	ND
Hypoxanthine-guanine phosphoribosyltransferase	BC_0071	Q81J83	20.2	−29.20	38	12	+	−	Cytosolic fraction
Superoxide dismutase Mn_2	BC_5445	Q814I6	23.9	−75.05	56	13	−	+	ND
Chaperon protein DnaK	BC_4312	Q818E9	65.6	−145.58	42	26	−	+	ND
GroES cochaperonin	BC_0294	Q814B1	10.0	−70.83	76	17	−	+	ND
Nucleoside diphosphatase kinase	BC_1515	Q81FQ4	16.6	−44.62	50	12	−	+	ND
ABC transporter ATP-binding protein	BC_4221	Q818M8	33.1	−39.27	34	15	−	+	ND
37°C									
Uncharacterized protein	BC_5391 (*gerQ*)	Q814N4	16	−38.90	30	15	+	−	Coats
Superoxide dismutase (Cu-Zn)	BC_4907	Q816F2	19	−34.89	47	11	+	−	ND
Uncharacterized protein	BC_0212 (*yusW*)	Q81IY1	17.6	−51.73	44	13	+	−	Coats
Alkyl hydroperoxide reductase C22	BC_0377	Q81IK9	20.6	−45.22	68	16	−	+	ND

[a] +, presence of protein in analyzed bands; −, absence of protein in analyzed bands.
[b] Proteins were identified by LC-MS/MS analysis, and the names correspond to the annotation in the *B. cereus* ATCC 14579 genome.
[c] Here, protein E value is the product of its valid unique peptide E values and is different from the protein E values determined by X!Tandem. The values are expressed in log (E value).
[d] Number of spectra in LC-MS/MS analysis.
[e] Location of proteins according to Abhyankar et al. (48). ND, not determined.

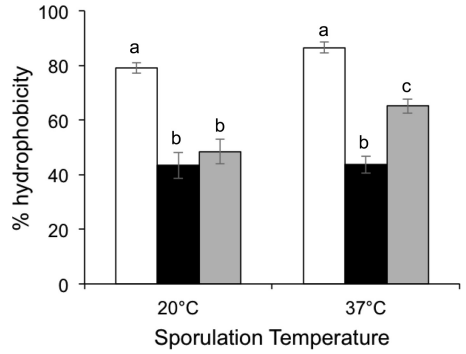

FIG 4 Hydrophobicity of spores of *B. cereus* ATCC 14579 WT (white bars), Δ*cotE* (black bars), and Δ*cotE*Ω*cotE* (gray bars) strains formed at 20°C and 37°C. The percentage of hydrophobicity represents the proportion (×100) of spores in *n*-hexadecane after a separation into solvent and water phases. Each hydrophobicity percentage is the mean from three replicate experiments, each performed with an independently prepared spore suspension. Bars represent standard deviations. Different letters (a, b, c) show significant differences at *P* values of <0.05 (Tukey's test).

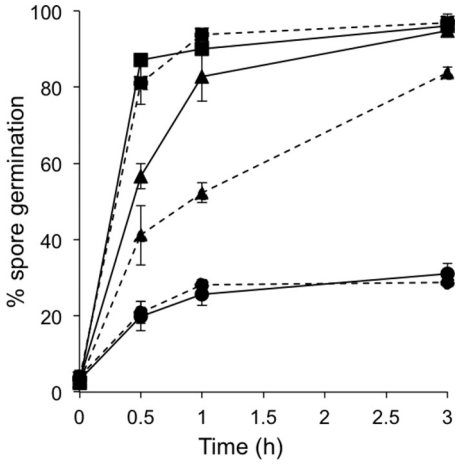

FIG 5 Germination in response to 0.5 mM inosine of *B. cereus* ATCC 14579 (■), Δ*cotE* (●), and Δ*cotE*Ω*cotE* (▲) spores formed at 20°C (solid line) and 37°C (dashed line). The percentage of germination was determined after counting of phase-bright (ungerminated) and phase-dark (germinated) spores with a phase-contrast microscope. The numbers represent the means from three replicate experiments, each performed with an independently prepared spore suspension. Bars represent standard deviations.

paired assembly of coat/exosporium components required for proper germination under specific conditions.

Sporulation temperature and deletion of *cotE* affect the resistance of spores to physical insults. Spores of the three strains formed at 20°C were less resistant to wet heat than spores formed at 37°C (Fig. 6A), confirming that sporulation temperature is an important factor influencing the wet-heat resistance of *B. cereus* spores. Surprisingly, whatever the sporulation temperature, Δ*cotE* spores tended to be more resistant to wet heat at 90°C than WT spores (Fig. 6A) and complementation restored the WT phenotype for both sporulation conditions. This result suggests that absence of CotE could directly or indirectly affect one or several of the factors known to influence wet-heat resistance, i.e., water, mineral ion, dipicolinic acid (DPA), cortex composition, or small acid-soluble protein (SASP) content of the spore core (4). One possibility is that CotE is required for the proper assembly of one or more components required for proper formation and/or modification of the spore cortex peptidoglycan.

Spores of the Δ*cotE* strain formed at 20°C and 37°C had a significantly higher sensitivity to UV-C than spores of the WT (Fig. 6B). WT spores formed at 20°C were more resistant to UV-C than those formed at 37°C (Fig. 6B). Spores of the Δ*cotE*Ω*cotE* strain had the same sensitivity as Δ*cotE* spores when formed at 20°C but had an intermediate resistance to UV-C, between the ones of WT and Δ*cotE* spores, when formed at 37°C (Fig. 6B).

When submitted to a pulsed-light (PL) treatment, Δ*cotE* spores formed at 20°C and 37°C were more sensitive than WT spores (Fig. 6C). The Δ*cotE*Ω*cotE* spores (reduction of 3 $\log_{10}$) had an intermediate resistance to PL, higher than or similar to that of the Δ*cotE* spores but lower than that of WT spores, suggesting that the higher PL sensitivity of Δ*cotE* spores was due mainly to *cotE* deletion. In addition, spores formed at 20°C and 37°C behaved similarly when exposed to PL.

Sporulation temperature and *cotE* deletion affect the chemical resistance of spores. Considering resistance to H_2O_2, Δ*cotE* spores formed at 20°C were significantly less resistant ($P < 0.05$) (34% of spores surviving after a 30-min exposure to 5% H_2O_2) than WT spores (56% surviving spores), and the same effect was observed with spores formed at 37°C (14% versus 72% for Δ*cotE* and WT spores, respectively) (Fig. 7A). Complementation partially restored the resistance of spores formed at 37°C (51% surviving spores) but not that of spores formed at 20°C (35% surviving spores), as no significant difference with Δ*cotE* spores was observed. Additionally, WT and Δ*cotE*Ω*cotE* spores formed at 20°C were less resistant to 5% H_2O_2 than those formed at 37°C, whereas Δ*cotE* spores were surprisingly more resistant (Fig. 7A).

Proper formation of the spore surface layers affords protection of the underlying spore cortex peptidoglycan against peptidoglycan-breaking enzymes (10). When analyzing the resistance of spores to lysozyme and mutanolysin, Δ*cotE* spores formed at 20°C were significantly more sensitive (9% and 7% surviving spores, respectively) than WT spores (82% and 73% surviving spores, respectively) after exposure to either enzyme for 60 min (Fig. 7B and C). Δ*cotE* spores formed at 37°C were also significantly more sensitive ($P < 0.05$) to both enzymes (79% and 64%, respectively) than WT spores (90 and 75%, respectively). Complementation restored resistance to lysozyme and mutanolysin to WT levels at both sporulation temperatures. The increased resistance of *cotE* spores to peptidoglycan-breaking enzymes is consistent with the TEM analysis and suggests that both the assembly of the coat and the assembly of the exosporium contribute to cortex protection.

DISCUSSION

We have shown that the sporulation temperature and deletion of *cotE* have a strong impact on the morphology and properties of *B. cereus* ATCC 14579 spores. For spores of the *cotE* mutant formed during sporulation either at 20°C or at 37°C, the exosporium, as seen by TEM, was present as fragments that failed to encase the spore and were often detached. Thus, CotE is required for proper assembly of the exosporium and its attachment to the inner layer structures of *B. cereus* spores, consistent with previous work on the characterization of a *cotE* deletion mutant of the related organism *B. anthracis* (30). At a sporulation temperature of 37°C, deletion of *cotE* had no detectable effect on the morphology of the *B. cereus* spore coat, while the coats of *B. anthracis* spores were not fully

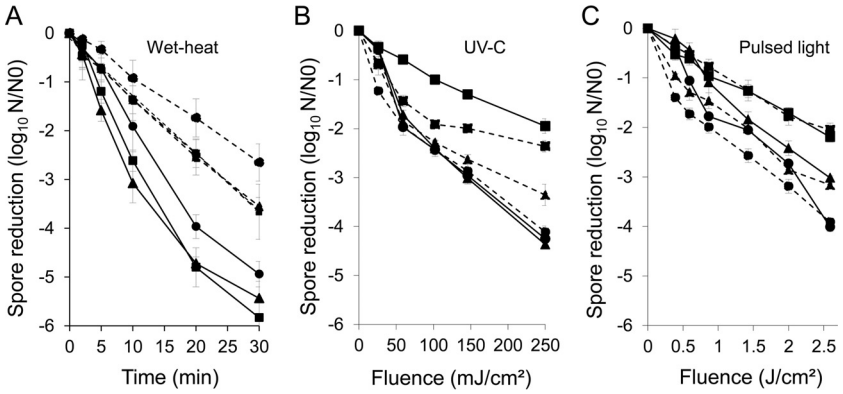

FIG 6 Sensitivity of *B. cereus* ATCC 14579 (■), Δ*cotE* (●), and Δ*cotE*Ω*cotE* (▲) spores formed at 20°C (solid line) and 37°C (dashed line) to wet heat at 90°C (A), UV-C (B), and pulsed light (C). Each log_{10} reduction is the mean from three replicate experiments, each performed with an independently prepared spore suspension. Bars represent standard deviations.

attached to the underlying cortex layer (23, 30). However, when formed at 20°C, in addition to impaired assembly of the exosporium, Δ*cotE* spores showed incomplete and often loosely attached coat layers. Thus, CotE plays an additional role in coat assembly at low sporulation temperature. Consistent with the ultrastructural analysis, the representation of several proteins in extracts of coat and exosporium proteins differed between WT and Δ*cotE* spores at the two sporulation temperatures tested. The alterations in the representation of these proteins are likely to contribute to the morphological changes observed. Importantly, deletion of *cotE* affects the presence or extractability of more proteins in spores formed at 20°C than in those formed at 37°C. CotE itself is more represented in extracts from spores produced at 20°C than in those produced at 37°C (Fig. 1). More CotE could be produced and assembled at low temperature, and we note that expression of the *B. subtilis cotE* and *sigE* genes is also known to be induced during adaptation to low temperature (54). It is also possible that the increased representation of CotE in spores formed at 20°C results from increased extractability. In *B. subtilis* spores, a fraction of CotE is present as multimeric species, possibly covalently cross-linked (19, 22, 55, 56). At least one multimeric form of CotE is extracted from *B. cereus* spores produced at either 20°C or 37°C (Fig. 1). However, this form is also more represented in the extracts from spores produced at 20°C. This suggests that the increased representation of CotE in the extracts from the spores formed at 20°C may result from increased synthesis of the protein. It was previously noted that lowering the sporulation temperature resulted in increased levels of the outer coat (CotE-dependent) CotA laccase in *B. subtilis* spores, and it was suggested that this could be due to changes in the level or activity of CotE (3). It is tempting to suggest that increased assembly and/or activity of CotE may be required to maintain proper assembly and function of the coat/exosporium in *B. cereus* when sporulation takes place at 20°C.

The profile of extractable coat proteins for WT or Δ*cotE* spores also suggests that the *cotE* deletion impacts the structure and composition of both the coat and the exosporium layers of the spore, with the effect on the coat more pronounced at 20°C. CotB, not detected in Δ*cotE* spores formed at 20°C, is a well-characterized exosporium orthologue of the *B. subtilis* outer coat protein CotB (34, 57, 58). Superoxide dismutase (SOD) proteins were also undetected in Δ*cotE* spores regardless of the sporulation temperature. At least one SOD is associated with the spore coat in *B. subtilis* (59). An iron/manganese SOD is also associated with the exosporium of *B. anthracis*, where it plays a role in oxidative stress resistance (50, 60, 61). That these proteins are absent from Δ*cotE* spores is consistent with the strong impact of the mutation in exosporium assembly and with previous work on the character-

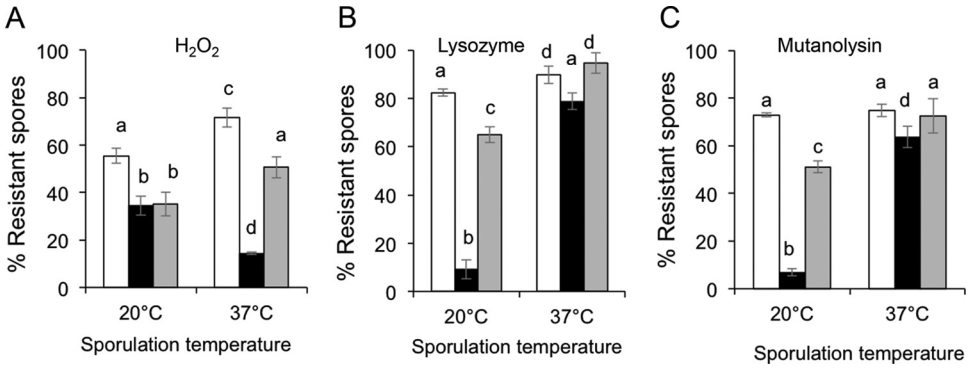

FIG 7 Resistance of *B. cereus* WT (white bars), Δ*cotE* (black bars), and Δ*cotE*Ω*cotE* (gray bars) spores formed at 20°C and 37°C exposed for 30 min to 5% H_2O_2 (vol/vol) (A), for 1 h to 7 U · ml^{-1} lysozyme (B), and for 1 h to 250 μg · ml^{-1} of mutanolysin (C). For each inactivation treatment, bars sharing the same letter (a, b, c, d) were not significantly different ($P < 0.05$, Tukey's test). Bars represent standard deviations for three experiments, each performed with an independently prepared spore suspension.

ization of *B. anthracis* Δ*cotE* spores (30). Δ*cotE* spores are susceptible to H_2O_2, and it seems possible that the impaired assembly of SOD (and possibly also of the manganese catalase CotJC; see below) contributes to this phenotype. *B. subtilis* spores with altered coats are more sensitive to oxidizing agents than those with intact coats, and the outer coat-associated CotA laccase was shown to have a role in protection against H_2O_2 (4, 62, 63). Importantly, a group of proteins affected by deletion of *cotE* may be associated with the coat. For instance, the BC_5391 protein, absent in Δ*cotE* spores formed at 37°C, is an orthologue of the *B. subtilis* coat protein GerQ, which is most likely localized in the inner coat (17, 64). Although deletion of *gerQ* (*ywdL*) in *B. cereus* leads to exosporium fragility, this effect could be due to an abnormal assembly of the underlying coat (12). Also undetected in *B. cereus* Δ*cotE* spores formed at 20°C is CotJC, previously detected in a proteomic analysis of *B. cereus* ATCC 14579 spores (48) and whose *B. subtilis* orthologue is a component of the innermost coat layers (65). Lastly, the BC_4419 protein, which is most likely an orthologue of the *B. subtilis* YhcN lipoprotein thought to be present in the spore inner membrane and to be important for spore germination (49, 55), is also undetected in *B. cereus* Δ*cotE* spores formed at 20°C. We do not presently know whether the proteins whose assembly is impaired by the *cotE* mutation at 20°C are directly recruited by CotE to the developing spore or whether CotE is required for their maintenance around the spore, as found for several *B. subtilis* coat proteins (16, 66). CotE may be localized at the edge of the coat, from where it somehow promotes attachment of the exosporium. In its absence, while assembly of the exosporium is compromised, several coat proteins may fail to remain associated with the spore. If so, why more proteins are missing from Δ*cotE* spores produced at 20°C is unclear. While it is possible that the missing proteins are directly recruited by CotE, it may also be that at 20°C the coat structure is not properly consolidated, for example, through covalent protein cross-linking, and more proteins are lost from these spores over time.

Importantly, the alterations seen in the composition of the surface layers of the Δ*cotE* spores may also explain, at least in part, additional properties of the mutant spores. Deletion of *gerQ* (*ywdL*) in *B. cereus* impairs germination in response to exogenous CaDPA and to inosine (12, 67). Deletion of *cotE* also impairs inosine-triggered spore germination in *B. anthracis* (30). Therefore, the *B. cereus* germination phenotype of the Δ*cotE* spores could be due, at least in part, to the misassembly of the YhnC/YlaJ orthologue at 20°C and of GerQ/YwdL at 37°C. Additional coat proteins, such as CotO and CotH, which play a role in germination, possibly by affecting the interaction of germinants with spores (11), were undetected in Δ*cotE* spores in our study. Moreover, the exosporium contains several enzymes, such as the alanine racemase Alr, shown to be involved in *B. anthracis* spore germination (68) and which was also undetected in Δ*cotE* spores in our study. Thus, the impaired interaction of germinants with spores and/or reduced accessibility to germinant receptors caused by the observed spore structural alterations may explain the impaired germination observed for Δ*cotE* spores formed at both 20°C and 37°C.

Also, Δ*cotE* spores showed lower hydrophobicity than WT spores. The exosporium is hydrophobic and plays a major role in spore adherence and pathogenicity of some species (69). Our results are also consistent with previous studies showing that *B. cereus* Δ*exsA* spores lacking an exosporium are less hydrophobic than WT spores (28). We did not observe any additional effect when lowering the sporulation temperature on the hydrophobicity of WT or Δ*cotE* spores of *B. cereus*, in line with previous results in other *B. cereus* strains and other *Bacillus* species (70).

When formed at 20°C, Δ*cotE* spores were much more sensitive than the WT spores to lysozyme and mutanolysin activities. Possibly, the defect in coat and exosporium assembly observed for Δ*cotE* spores formed at 20°C increases the accessibility of both lytic enzymes to the cortex. This is in line with the observation that *B. subtilis* Δ*cotE* spores, which fail to assemble the outer coat layer, are sensitive to lysozyme (23). However, we cannot exclude that *cotE* expression is required for the assembly of components involved in proper cortex formation and/or maturation, independently from or in addition to a barrier function of the coats (19). Interestingly, Δ*cotE* spores were more resistant to wet-heat treatment than WT spores at the two tested sporulation temperatures and even more when formed at higher temperature. In contrast, increased resistance to wet heat correlated with higher sporulation temperatures for *B. subtilis* spores (3). However, some environmental conditions such as anaerobiosis are known to lead to increased heat resistance of *B. cereus* spores (71). Heat resistance is multifactorial, and a number of factors, such as a higher quantity of DPA, lower spore water content, or the structure of the cortex, could explain the higher heat resistance of Δ*cotE* than WT spores (3, 4). *B. cereus* Δ*cotE* spores are less resistant to UV radiation (delivered by either UV-C or PL) treatment than WT spores. In contrast, spores of a *B. subtilis* Δ*cotE* mutant were more resistant to UV-C treatment (63). The forespore-specific α/β-type SASPs are the major determinant for spore resistance to UV radiation in *B. subtilis* (72), and it is presently unknown whether CotE somehow influences their level (63). Lowering the sporulation temperature did not impact the resistance to UV of WT and Δ*cotE* spores in the *B. cereus* strain ATCC 14579, in line with the observation that *B. subtilis* strains prepared at different temperatures had identical levels of α/β-type SASPs and essentially identical resistance to UV radiation (3). However, lowering the sporulation temperature caused a reduction in the resistance of *Bacillus weihenstephanensis* (belonging to the *B. cereus* group) strain KBAB4 spores to UV radiation (45). The reason for these differences among strains is presently unclear. Possibly, as-yet-unknown factors contribute to spore resistance to UV radiation. In conclusion, low sporulation temperature revealed a new major role for the CotE protein in spore morphogenesis of *B. cereus*. Understanding the spore assembly mechanisms under different environmental conditions may help to assess the structure and properties of spores existing in the natural environment and the development of optimized industrial treatments, particularly in the food chain, to limit the survival and germination of contaminating spores.

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